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ORIGINAL ARTICLE

Decrease in Barbaloin and Product of Butyric acid Fermented by Endophytic Bacteria in Aloe Arborescens leaves

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ABSTRACT

Quantitative analysis of phenolic compounds in *Aloe arborescens* fermented extract revealed that barbaloin concentration was found to be 14 µg/mL (14 ppm) by HPLC analysis. Seven phenolic compounds could be identified: aloesin, 8-C-glycosyl-7-O-methyl-S-aloesol, aloenin, isobarbaloin, barbaloin, 2'-O-feruloylaloenin, and

aloe-emodin based on previous HPLC analysis. Short chain fatty acids such as acetic, propionic, butyric, lactic acid, were identified in the fermented extract of *A. arborescens* by GC/MS analysis. Reduction of barbaloin concentration and production of butyric acid clearly indicate a potential efficacious role of endophytic bacteria in *A. arborescens* leaves. Dietary intake of the extract fermented by endophytic bacteria in *A. arborescens* highlights immune-stimulating activity to human health and strongly influences an importance of host-microbial crosstalk to ensure maintenance of homeostasis.

Key words: *Aloe arborescens*; Endophytic bacterial fermentation; Barbaloin; Short chain fatty acids

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INTRODUCTION

Aloe arborescens is native to southern Africa and is a vernacular name of shrubby aloe. In Japan where it was first introduced in the 17th century, it became naturalized. The taxonomy of *A. arborescens* is complicated by occurrence of interspecific hybrids both in the wild and in cultivation. Chromosome number of *A. arborescens* is 2n = 14 and it is a morphologically very variable species^[1,2]. On *A. arborescens* var. *natalensis* (kidachi aloe) in Japan, Reynolds^[3] concluded that it is regarded *A. arborescens* as being a variable species, than to attempt to uphold varietal names (*A. arborescens* var. *natalensis*) on overseas growth forms. Furthermore, Reynolds suggested that this is presently the best approach to allow for natural variation found within and between populations of *A. arborescens*.

A 2-year carcinogenicity study of *A. arborescens*, a health-food additive in Japan, was conducted by Yokohira *et al*^[4] for assessment of toxicity and carcinogenic potential in the diet at doses of 4 or 0.8% in groups of male and female Wistar Hannover rats. Both sexes receiving 4% *A. arborescens* showed diarrhea, with loss of body weight gain. The survival rate in the 4% female group was

significantly increased compared with control females after 2 year. Adenomas or adenocarcinomas in the cecum, colon, and rectum were observed in 4% male but not in the 0.8% and control male groups. Similarly, in females, adenomas in the colon were also observed in the 4% but not 0.8% and control groups. *A. arborescens* used as a food additive, exerted equivocal carcinogenic potential at 4% high-dose level on colon in the 2-y carcinogenicity study in rats. *A. arborescens* is not carcinogenic at nontoxic-dose levels and that carcinogenic potential in at 4% high-dose level on colon is probably due to irritation of the intestinal tract by diarrhea. Furthermore, the authors demonstrated that the no observed adverse effect level (NOAEL) for *A. arborescens* was 0.16% in diet, which is equivalent to 87.7 and 109.7 mg/kg/day in males and females, respectively^[5]. Toxicological study of barbaloin (aloin) was performed by Boudreau *et al*^[6]. Groups of 10 male F344/N rat were administered aloin at 0, 6.95, 13.9, 27.8, 55.7, 111, 223 and 446 mg/kg drinking water for 13 weeks. Aloin indicated dose-related increased incidences and severities of mucosal and goblet cell hyperplasia that extended from the cecum to the rectum, with increased incidences and severities detected at aloin doses ≥ 55.7 mg/kg drinking water. International Aloe Science Council (IASC) certified *Aloe vera* products from gel for oral consumption are analytically tested for aloin content to ensure compliance with a maximum limit of 10 parts per million^[7]. FDA issued a final rule stating that the stimulant laxative ingredients of aloe whole leave in OTC drug products are not generally recognized as safe or are misbranded^[8]. The German Federal Institute for Risk Assessment (BfR: Bundesinstitut für Risikobewertung) has ruled whole-leaf aloe preparations: *A. arborescens* preparations, containing anthranoids not suitable for use in foods, including food supplements^[9].

In previous HPLC analysis, phenolic compounds in *Aloe* species indicated that the content of phenolic compounds increases clearly in the order: base, center and point, suggesting the relation to the part of the leaf decreases with age in aloe. The content per fresh weight of inner epidermal layer was 1.25-3 times higher than those of gel^[10]. Li *et al*^[11] reported relationship between leaf structure and aloin content, and aloin content in leaves of *A. arborescens* is three times higher than that of *A. vera*.

The stability and degradation products of aloin A, one of barbaloin isomer, under various pH, temperature and light conditions usually encountered in processing was explored by Ding *et al*^[12]. The stability of aloin A was significantly affected by temperature and pH. Aloe-emodin, elgonica-dimers A and B (aloe-emodin-aloin A and B dimers) were characterized as major degradation products of aloin A at pH 5.0 or below, and elgonica-dimers were mainly formed at 4°C as well. 10-Hydroxyaloin A and B were found under any condition except at pH 2.0 and 3.0, and they were mainly formed under high temperature, neutral-basic and any light conditions. Shindo *et al*^[13] investigated the contents of barbaloin, isobarbaloin, aloin-dimers A, B, C, D and aloe-emodin in aloe drinks. When aloe drinks were stored for 4 weeks at 5°C after opening the bottle, the decrease of barbaloin and isobarbaloin were observed by using of LC-MS analyses, suggesting that barbaloin and isobarbaloin in aloe drinks is converted to the dimer and then to the trimer during storage.

Chiodelli *et al*^[14] evaluated the effects of different extracts of *Aloe vera* and *A. arborescens* in fermented milk, taking into account both the prebiotic effect of aloe polysaccharides. The results showed a beneficial effect of 5% aloe inner gel on growth of lactic acid bacteria, *Lactobacillus* spp. Bourriaud *et al*^[15] assessed the role of D- and L-lactate as a precursor for butyrate biosynthesis in human colonic microbiota. The study suggests that the butyrogenic

capability of colonic prebiotics could be related to lactic acid availability. The human intestinal microbiota can utilize both D- and L-lactate as precursors for butyrate synthesis. Inter-individual variation was found, suggesting the presence of the lactate-utilizing bacteria in the human intestinal microflora. Munoz-Tamayo *et al*^[16] provided a mathematical model to analyze the production of butyrate by lactate-utilizing bacteria from the human colon. The findings of the mathematical model adequately matched those from the bacterial batch culture experiments.

In previous paper we described that aloe vera gel with endophytic bacteria, *Bacillus cereus*, *B. licheniformis* and *Lactobacillus paralimentarium*, produced butyric acid, and suggested that daily consumption of *Aloe vera* gel juice may be beneficial to putative prophylaxis for health and QOL as an immune modulation^[17,18]. Furthermore, Al-Madboly *et al*^[19] investigated that the probiotic activity of *Aloe vera* in *in vitro* fermentation with *L. fermentum* isolates provided acetic, propionic and lactic acid. Li *et al*^[20] investigated that *Bacillus* spp. DU-106, a newly isolated member of *B. cereus* group, exhibits the predominant ability to produce L-lactic acid. Fakhry *et al*^[21] found that sequence comparisons between human gut intestinal tract and plant root isolates using multiple genes, implying that human and plant isolates are closely related strains. The authors suggested that the isolated *Bacillus* species do not transit, but rather colonize this specific habitat, such as sporulate in anaerobic conditions, and proposed them as probiotic strains for human use. Furthermore, Torres *et al*^[22] analyzed a subset of probiotic microbes in genus *Bacillus* to evaluate the possibility that they may move from plant to human hosts after consumption of food plants. Sequence comparisons between human and plant root isolates using multiple genes indicate that human and plant isolates are closely related strains. Consumption of microbes in plants may be one way the human gut microbiome becomes established. Potential efficacious role of barbaloin, aloe-emodin and short chain fatty acids fermented of *Aloe vera* and *A. arborescens* needs further scrutiny and evidence-based documentation^[23].

Present investigation aims to evaluate the concentration of barbaloin by HPLC/UV, and production of short chain fatty acids by GC/MSD analysis for the extract fermented by endophytic bacteria in *Aloe arborescens* leaves.

MATERIALS AND METHODS

Aloe arborecens sample preparation

Aloe arborescens leaves collected from the herbal garden of Fukuyama university. *A. arborescens* leaves, 300g, were washed with 0.02% hypochlorite solution and rinsed with water. After trimming off butts and tips, the whole leaves were cut into pieces containing 20% W/W sucrose in closed bottle at room temperature for one month. The pH of exuded filtrate, 220ml, was 3.38-3.42 at 19.4-12.0°C in Oct.-Nov. 2017. The solution is pale color with no bitter taste. The fermented extract was kept in a cool closed bottle until use.

HPLC/UV detection of phenolic compounds in *Aloe arborescens* leaves

Determination of phenolic compounds in *Aloe arborescens* by HPLC/UV detection

Materials: Acetonitrile of HPLC grade was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was double distilled using Automatic Water Still (Sci Finetech, Seoul, South Korea). Standard barbaloin and aloe-emodin were purchased from Wako.

Instrumentation: The separation was performed on an Ultimate 3000 rapid separation instrument (Dionex, Sunnyvale, CA, USA) equipped with a VWD-3400RS UV detector and a WPS-3000TPL RS auto-sampler, Chromeleon[®] software (Ver.7.2) was employed for data processing. The column was X-Bridge[™] C18 (250 mm × 4.6 mm, 5 μm particle size, Waters Corp., Milford, USA) and the detector was set at $\lambda = 293$ nm.

Stock solutions: Barbaloin, 1.9mg, were weighed, transferred into a 10mL volumetric flask, dissolved in 3 mL methanol and completed to volume with methanol (190 μg/mL). Mixed standards of phenolic compounds, 10mg, were weighed, transferred into a 10mL volumetric flask, dissolved in 5 mL methanol and completed to volume with methanol (1 mg/mL).

Methodology

HPLC conditions: Mobile phase; acetonitrile/water (50:50, v/v), Chromatographic mode: gradient elution (12 to 50% acetonitrile in 50 min), Flow rate; 1 ml/min, Injection volume 10 μL, column; Xbridge[™] C18 (4.6 × 150mm, 5 μm), column temperature; 35°C, Detector; UV at 293 nm.

RESULTS

Quantitative results of phenolic compounds in *A. arborescens* fermentation extract revealed that the amount of barbaloin was found to be 14 μg/mL (14ppm). Based on previous analysis, seven phenolic compounds could be identified: aloesin; 8-C-glucosyl-7-O-methyl-(S)-aloesol; aloenin; isobarbaloin, barbaloin; 2''-O-feruloylaloenin and aloee-modin. The extract does not contain any detectable amounts of aloeresin A, isoaloesin D, or aloeresin E^[10].

GC-MS analysis

Sample preparation: The ether extract of the fermentation broth, 0.1g, was dissolved in 2ml hexane, sonicated for 10 minutes, methylated by sodium methoxide, then clear hexane layer filtered through PTFE membrane. Sample was diluted 1:50 with hexane before injection under SIM conditions.

Instrument used: GC/MSD 5977A, Agilent, USA. The column used: Agilent, DB 225 ms 60m × 250μm × 0.25 μm. Oven program: 35°C for 3.7 min, then 7°C/min to 150°C. Inlet: Split mode. Split ratio 25:1. Liner Agilent 5190-2294: 990μL. Inlet temperature: 100°C for 1 min, then 100°C/min to 225°C. Auxiliary temperature: 230°C.

MS information:

Acquisition mode: SCAN; Scan parameters: Low Mass 45, High Mass: 550.00

Environmental condition: 24°C, humidity: 51%.

SIM Parameters: illustrated in the table 1.

Results

The fermentation extract of *Aloe arborescens* leaves exhibited the production of acetic, propionic, butyric, and lactic acid as short chain fatty acids (SCFAs) by GC/MSD analysis under SIM mode. The concentration of SCFAs was acetic acid (0.024%), lactic acid

(19.12%), propionic acid (0.01%), and butyric acid (0.15%).

DISCUSSION

The interaction of endophytic fungi with host plants results in a compromise between mutualism and antagonism to create a harmonious symbiotic system. In the previous papers we described the benefits of endophytic bacteria producing butyric acid in *Aloe vera* leaves^[17,18]. Asai *et al*^[24] reported new short-branched fatty acid dimers in the cultures of endophytic fungus, *Mycosphaerella* sp., isolated from *Aloe arborescens* leaves. As biological active constituents of leaves of *A. arborescens*, Hirata and Suga^[25] found several bioactive substances, such as magnesium lactate, succinic acid, aloenin, aloee-modin, and barbaloin. Magnesium lactate inhibits histidine decarboxylase, which prevents the formation of histidine. This may partially explain the antipruritic and anti-inflammatory effect of *A. arborescens*.

In the present study, we found that acetic, propionic, butyric, and lactic acid were produced from the fermentation extract of *A. arborescens* leaves with endophytic bacteria by GC/MSD analysis. Fermented extract of *A. arborescens* leaves exhibited a possible promotion of the growth of host plant to achieve a balanced living environment by Josi *et al*^[26]. It is important to note that endophytic bacteria in *A. arborescens* produced short chain fatty acids (SCFAs), such as acetic, propionic, butyric, and lactic acid in high concentration in *in vitro* fermentation extract. Butyric acid is preferred energy source for colonocytes and plays an important role in gastrointestinal tract homeostasis. Production ratio of endophytic bacterial metabolic SCFAs, butyric acid, in *A. vera* and *A. arborescens*, which direct the butyrogenic capability of colonic prebiotics related to lactate availability^[15], could be suggested as one of the metabolic difference between *A. vera* and *A. arborescens*, because magnesium lactate was isolated from *A. arborescens* leaf^[25]. Some ingredients, such as aloenin and lactate, in the extract of *A. arborescens* antagonize or synergize with each other to produce a harmonizing efficacy between wound-healing and anti-inflammatory activity through an immunoreaction. On the study of *A. arborescens* leaf pulp, we isolated a partially acetylated β-D-mannan, named aloee-mannan having molecular weight: 15,000 by equilibrium ultracentrifugation as a pure state. The water-soluble partially acetylated β-D-mannan (aloe-mannan) exhibited the inhibiting activity for the implanted salcoma-180 in mouse^[27]. A homogeneous glycoprotein (Mw: 40,000) containing 50.7% of protein showed bradykinin-degrading activity^[28], DNA synthesis stimulation, and lectin properties^[29]. Clinical trial of *A. arborescens* extract^[30] demonstrated that the active compounds, such as glycoprotein, clearly enhance activity of both phagocytosis and NBT reduction, and polysaccharide act as an immune-potentiator and promote phagocytosis in adult bronchial asthmatics. The actual phagocytosis step is really a cleaning up operation after some of the earlier immune processes have taken place. This process of phagocytosis plays an important part of the overall process of immunity. Randomized study of chemotherapy versus bio-chemotherapy with chemotherapy plus *A. arborescens* in 240-patients with metastatic cancer was demonstrated by Lissoni *et al*^[31]. The study suggests that *A. arborescens* may be successfully associated with chemotherapy to increase its efficacy in terms of both tumor regression rate and survival time.

Endophytic microbial-derived butyric acid in *A. arborescens* leave could exert anti-cancerous effects by several mechanisms, highlighting the importance for health maintenance^[32]. Regulation of the epigenome in particular inhibition of histone deacetylases,

Table 1 Short chain fatty acids identified by GC/MSD analysis under SIM mode.

	RT (min)	SIM Ions m/z	Conc. (g/100g)
Acetic acid methyl ester	12.492	43, 45, 60	0.024
Lactic acid methyl ester	13.56	45, 104	19.12
Propionic acid methyl ester	14.709	45, 73, 74	0.01
Butyric acid methyl ester	16.807	60, 73, 88	0.15

such as butyric acid, impacts pathogenic mechanisms involved in chronic disease. Kim *et al*^[33] demonstrated that probiotics mixture and sodium butyrate increased Th1 and Treg cell differentiation in mesenteric lymph nodes and spleen tissues in mouse model. These results suggest that the probiotic mixture and sodium butyrate can prevent and alleviate allergic symptoms.

Monthly variations and different parts in barbaloin content of *A. arborescens* were studied by Beppu *et al*^[34]. The barbaloin content in the supernatant of homogenized leaves showed 294.8 ± 24.31 ppm in average annual value and 288.5ppm in apex part on the same stalk. Barbaloin content in methanol supernatant of homogenized leaves of *A. vera* and *A. arborescens* showed high in *A. arborescens* (600 ppm in fresh tissue) and low in *A. vera* (400 ppm in fresh tissue)^[10]. In order to remove of barbaloin, various treatments are utilized: the active charcoal adsorption process and cellulase process for viscosity reduction in industrial processing. On the microbiology of *A. vera* and *A. arborescens*, one should be cognizant of two sources: endogenous and exogenous microbiota. Endophytes, endogenous microorganisms, include bacteria and fungi living within plant tissues without causing any overt negative effects. Barbaloin content in the fermented extract by endophytic bacteria in *A. arborescens* was reduced to 14ppm in present experiment. It is important to note that endophytic bacteria in *A. arborescens* provided 14ppm barbaloin in *in vitro* fermentation extract, which shows near concentration to IASC carcinogenetic activity standard (less than 10ppm of barbaloin). IASC certified that aloe vera products for oral consumption are analytically tested for barbaloin content to ensure compliance with a maximum limit of 10 ppm, and aloe vera products made to IASC standards for barbaloin content are safe^[35]. The concentration of barbaloin 14ppm in original fermented extract in *A. arborescens* could be diluted to less than 10 ppm when it is used as aloe soft-drinks and beverage.

Dietary intake of extract fermented by endophytic microbiota in *A. arborescens* leaves provides for beneficial influences to human health maintenance. Further study is needed to identify the endophytic microbiota in the fermented extract of *A. arborescens*.

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